Nucleoporin 153: Engineering a mammalian expression vector to study its biological role

Dissertation Submitted for the Award of the Degree of Master of Philosophy in Biochemistry

By

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UNDER THE SUPERVISION OF

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CERTIFICATE

Certified that the work in the dissertation entitled**"Nucleoporin153: Engineering a mammalian expression vector to study its biological role"** has been carried out by Mr. Arif Bashir under the supervision of Dr.Shaida Andrabi (Department of Biochemistry,University of Kashmir) and the work is suitable for the award of M.Phil. Degree in Biochemistry.

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DECLARATION

I, Arif Bashir declare that the work embodied in this dissertation entitled **"Nucleoporin153: Engineering a mammalian expression vector to study its biological role"** has been carried out by me in the Department of Biochemistry, University of Kashmir, Srinagar and is original. The work embodies the results of my observations which are advancement to the previous knowledge in the subject.

Place: Srinagar **Arif Bashir**

Date: 17-04-2013

Dedicated to my parents

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All praises are due to Allah (SWT), exalted in might, the most merciful and beneficial. May Allah (SWT) send his mercy and blessings to prophet Muhammad (S.A.W), to his family, to his companion and to those who follow him? Knowledge and research is a precious gift of Allah (SWT) as the knowledge has been described as 'Light' in the Ultimate book of fate , Holy Quran, "Thus has come to you light from Allah''.

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BSA	Bovine serum albumin
cDNA	complementary de-oxy ribonucleic acid
DNA	De-oxy ribonucleic acid
EDTA	Ethylene diamine tetra-acetate
EtBr	Ethedium bromide
gm	Gram
kb	Kilo base
kDa	Kilo dalton
ml	Milli litre
µl	micro litre
PCR	Polymerase chain reaction
PIPES	Piperazine sulphanilic acid
RNA	Ribo nucleic acid
RNAse	Ribonuclease
Tm	Melting Temperature

Compartmentalization is the hallmark of eukaryotic cell characterized by the presence of DNA as genetic material inside the nucleus. Restricting the accessibility of large cytosolic proteins to the nucleosol with the barrier of nuclear envelope (NE) equipped with nanopore channel formed by NPCs (Nuclear Pore Complexes) which acts as gatekeeper between the two boundaries. Eukaryotes have achieved a complexity in transcriptional regulation not found in prokaryotic cells. Furthermore, the NE also serves as additional levels of regulation of gene expression e.g selective export of newly synthesized mRNA into the ribosome as well as the epigenetic control on gene expression and the establishment of higher levels of organization of the nuclear genome. Furthermore, NPCs being itself a supra-molecular complex of different nucleoporins gets formed in orderly orchestrated events during cell cycle. NPCs have been attributed with different functions ranging from transport of viral proteins to age dependent cell deterioration. NUP153 is an essential, indispensible and dynamic component of NPCs. It is a 153 kDa glyco-protein and in association with other nucleoporin proteins forms a stable 3D supra-molecular assembly within the bilayer nuclear membrane. NUP153 has been found to occur peripherally towards the nucleoplasmic side of nuclear membrane. NUP153 has been implicated in so many cellular processes ranging from the transport of viral proteins to the activation of apoptosis cascade in cells. We successfully cloned our gene of interest in two mammalian expression vectors viz pcDNA3 and pCMV-HA. We generated these two constructs to see the role of NUP153 in the transport of protein phosphate 2A (PP2A) (Normally, a resident of cytosol) and small T (ST) of Polyoma virus into the nucleus as our prospective study.

Cell biology faced so many revolutions from the Robert Hook's Micrographia published in 1665 in London to Rudolf Virchow, a German physiologist who in 1858 stated that all cells arise from pre-existing cells. With the emergence of eukaryotic cells, there occurred the division of labour and of course the compartmentalization, which is the hallmark of eukaryotic cells. Multicellular organism permits division of labour through specialization. The division of labour increases the operational efficiency of the eukaryotic organisms. Nuclear envelope (peri-nuclear cisterna) enclosing the DNA inside the nucleus forms one of the exquisite compartments of eukaryotic cells, which is formed of two concentric unit membranes, each 5-10 nm thick. The spherical inner membrane contains specific proteins that act as binding sites for the supporting fibrous sheath of intermediate filaments (IF) called nuclear lamina. The inner membrane is surrounded by the outer membrane which closely resembles to that of the endoplasmic reticulum and is continuous with the outer membrane. The space between the inner and outer nuclear membrane, known as the peri-nuclear space, is a fluid filled compartment, which contains fibrous, crystalline deposits, lipid droplets or electron dense material. The nuclear envelope in all eukaryotic forms, from yeast to humans is breached by nuclear pores. Nuclear pore accounts for 5-15% of the surface area of nuclear membrane in eukaryotic cells. It may be as high as 20-36% in terms of surface area as seen in amphibian oocytes, protozoa and certain plant cells. The number of nuclear pores breaching the nuclear membrane (Nuclear Pore Density_ NPD) has been seen to correlate with transcriptional activity of the cells. Nuclear pore density can be as low as 3 pores/μm^2 as seen in mature RBC and lymphocytes. These mature cells have the hallmark of non-proliferativity and metabolic inactivity. The majority of the proliferating cells have pore density lying between 7-12 pores/ μm^2. It has been reported that some hormones increase the density and number of nuclear pores (Miller *et al*., 1991). In non-germinal/ somatic cells, the nuclear pores are evenly distributed over the surface of nuclear envelope. However, the pore arrangement in other cell types is not random but ranges from rows as in spores of Equistem to clusters in oocyte of Xenopus laevis to hexagonal in malpighian tubules of Leaf hopper.

1.1 Nuclear Pore Complex (NPC):

Nuclear pore is composed of nuclear pore complex (NPC) proteins, which are supra-molecular assemblies of over 30 different types of nucleoporin (Nup) proteins. NPCs appear circular in surface view and have a diameter in between 10-100 nm. Previously, it was believed that a diaphragm made of amorphous to fibrillar material extends across each pore, limiting the free transfer of material. Such diaphragm called annulus has been observed in animal cells, but is lacking in plants. Recent electron microscopy has revealed that a nuclear pore has far more complex structure called nuclear pore complexes (NPCs). Each pore has an estimated molecular weight of about 50-100 MDa, depending upon the type of species. Negative staining techniques have demonstrated that the pore complex has an 8 fold or octagonal symmetry. NPCs are mainly made up of four different types of nucleoporins, the scaffold (NUP107, NUP160), transmembrane (pom 121, Gp210 and Ndc1), structural and peripheral nucleoporins (NUP153) (See Table 1). Interestingly, nucleoporins are equipped with limiting set of domains ranging from α solenoids, trans-membrane domains, coiled-coiled structures, β propeller and phenylalanine-glycine repeats (FG) (Alber *et al*., 2007 and Schwartz *et al*., 2005).

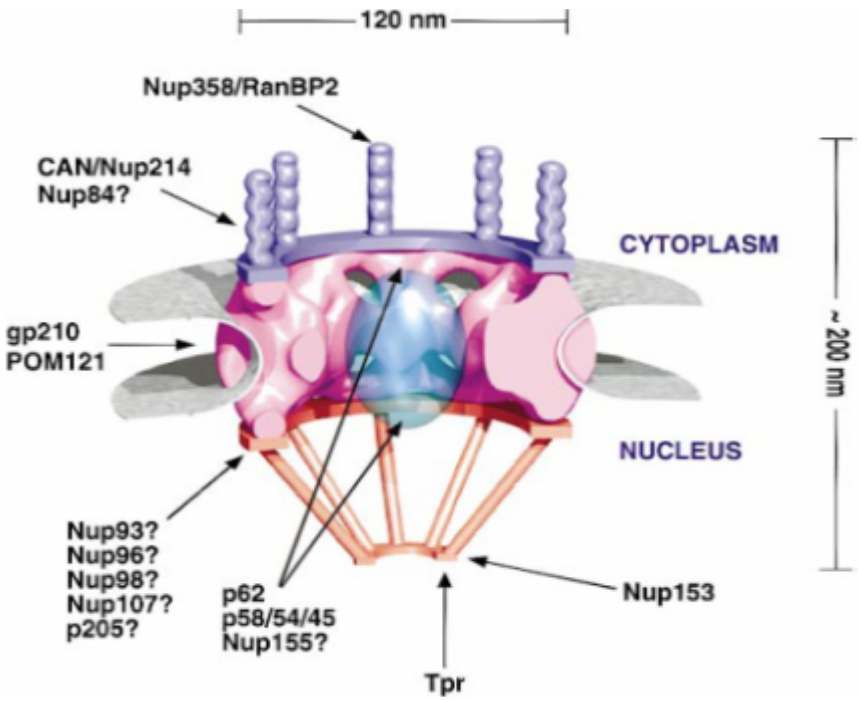

Fig 1: Picture showing nuclear pore complex (NPC).
(Adopted from **Cell**, 1999, **Vol**: 99, 677-690)

Table 1: Bp, β propeller; As, α solenoid; FG, Phenylalanine Glycine repeats; CC, coiled coil; ND, not determined, TMH, Transmembrane helical (Hetzer et *al.,* 2005).

	Vertebrate Nups andNupSubcomplexes	**Yeast Nups and NupSubcomplexes**	**Order of Assembly**	**Dynamics**	**Structural Folds**	**Predicted Function/s**
Cytoplasmic Face	Nup358	—	Early	ND	FG	Structural and Transport
	Nup214	Nup159	Middle	Stable	Bp, CC, FG	Structural
	CG1	Nup42	ND	Intermediate	FG	Structural and Transport
	Nup88	Nup82	Middle	Stable	Bp, CC	Structural
Symmetrically Distributed	Nup62 complex	Nsp1 complex	Middle	Intermediate	FG, CC	Structural and Transport
	Nup107-160 complex	Nup84 complex	Early	Stable	Bp, As	Scaffold
	Nup93 complex	Nic96 complex	Early	Intermediate	Bp, As, FG	Structural and Transport
	Rae1/Gle2	Gle2	ND	Dynamic	Bp	Transport
	Nup98	Nup100, Nup116 and Nup145N	ND	Dynamic	FG, Nup98 fold	Transport
Transmembrane	Pom121	—	Early	Stable	TMH, FG	Structural
	gp210	Pom152	Late	Dynamic	TMH	Transport
	Ndc1	Ndc1	ND	ND	TMH	Structural
	—	Pom34	ND	ND	TMH	Structural
Nuclear Face	Nup50/Npap60	Nup2	ND	Dynamic	FG	Transport
	NUP153	**Nup1**	**Early**	**Dynamic**	**FG**	**Structural and Transport**
	TPR	Mlp1 and 2	Late	ND	CC	Structural and Transport

The FG repeats of the nucleoporins, more particularly the peripheral nucleoporins, serve as the docking site for the import receptors. It has been studied that there is an orchestrated hopping of receptor-cargo complex on FG repeats of peripheral nucleoporins to traffickle through the interior of NPC. The FG repeats surrounding the lumen of the nuclear pore are thought to interact with the adjacent nucleoporins via weak hydrophobic interaction. FG-Nups are thought to play an important role of the permeability barrier of the NPC and secondly, they temporarily interact with soluble nuclear transport receptors and mediate the translocation of biomolecules across the NPC. Nuclear pores have been attributed with the function of acting as entropic bristle which means that the filaments of peripheral nucleoporins reduce the limited space to get an access through the nano-pore channel. Thus peripheral nucleoporins create an entropic barrier which decreases the probability of the molecules entering the pore without physically repelling them. To get an access through this available dimension of the pore, a molecule would have to reduce its entropy which is thermodynamically an unfavorable process ($\Delta G= \Delta H- T\Delta S$; Gibbs free energy concept).

1.2 Role of NPC in nucleo-cytoplasmic transport of biomolecules:

NPCs play a very important role in the transport of biomolecules, during passive as well as in active transport. For example, molecules smaller than 40 kDa readily diffuse through the NPC passively, obeying the Fick's law of diffusion, while biomolecules bigger than 40kDa (e,g mRNA, tRNA, catalytic and regulatory proteins etc.) need to be actively transported that requires ATP. The NPC pore appears to function like a close fitting diaphragm that opens when activated by signals. It is already well known that karyophillic proteins have nuclear import signal/ nuclear localization signal (NLS) which enables them to accumulate in the nucleus of the cell. The karyophillic signal for such a nuclear import apparently resides in the tail domain and its transport in the nucleus is an active process utilizing ATP as source of energy. Kinetic investigation has revealed that 1000 translocation occurs per second per NPC. The transport receptor interaction with the FG repeats of the nuclear pore enables large molecules to overcome the energy loss and trafficking through the entropic barrier.

It is important to note-down that in the selective-phase model of the nucleoporin, the formation of the sieve-like meshwork would also give rise to the formation of an entropic barrier for those molecules that can enter the nuclear pores of the FG network.

This entropic barrier would decrease the probability of the molecules entering the pores but is not able to define the permeability limit/ exclusion limit of the NPC, which could be elucidated by the meshwork nuclear pore size. Although these findings give an indication towards the FG network as the prime mechanism for pore selectivity, they are not yet enough to completely reject other proposed models for NPC permeability (Rout *et al*., 2003).

Classical nuclear import of many proteins in to the nucleus requires specialized transport machinery, which includes the adapter proteins e.g. importin α and importin β1. In association with each other, they form a dimeric complex that recognizes classical nuclear localization sequences in proteins destined for nucleus in the cytosol of the cell. Once the cargo is delivered to the nuclear side, binding of RanGTP to importin- β1 distorts the whole trimeric supramolecular complex, which ultimately leads to their dissociation from each other. Subsequently, importin α is exported back to cytosol by CAS (Cell Apoptosis Susceptibility) protein. NPCs being the prime constituent of nuclear pore provide the essential docking site for the translocation of import and export complexes. Defects in the classical nuclear import in humans due to oxidative stress on NPCs give rise to pathogenesis of several diseases ranging from cardiovascular disease to neurodegenerative disorder like Alzheimer's disease. Oxidative stress has also been investigated to affect many intracellular processes including classical nuclear import which consequently leads to cell death. The molecular mechanism that links oxidative stress to nuclear import is yet in its infancy (Mohamed Kodiha *et al*., 2008).

NUP153 contains separate binding sites for importin α/β, which acts as a mediator in classical NLS import. NUP153 having the importin binding site act as a dominant negative inhibitor of NLS import but has as such no effect on transportin mediated import of the bio-metabolites. The interaction of NUP153 with transportin distorted by a non-hydrolysable form of GTP or mutant of Ras having GTPase deficiency was not seen to affect the transportin carried cargo and export receptor Crm1 at the nuclear rim (Sara Nakielny *et al*., 1999).

Investigations have revealed that NUP153 interacts with wide variety of biomolecules like Ran, importin-α2, importin-β, transportin-1, exportin-1 (crm1), RanBP5, elF5A of RNA transport system, RNA, DNA and Tpr in the NPC (Nil Schrader *et al*., 2008).

In an attempt to see the interaction of FG repeats of various Nups in protein transport, an RNAi screening was performed (Nafiseh Sabri *et al*., 2007). Inducible S2 cells expressing cNLS-GFP and cNES-GFP constructs were transfected in cells and subsequently cNLS-GFP was seen in the nucleus and cNES-GFP was seen in the cytosol. To test whether cNLS-GFP and cNES-GFP are cargoes of importin α/βs and CRM1, they introduced dsRNAs against the Drosophilia homologues of importin α1, α2, α3, and β or kap β3. Only the addition of dsRNA against α3 and β importin RNAs reduced the relative level of cNLS-GFP, while the cNES-GFP level in cytosol remained unaffected of this dsRNA mediated silencing. Furthermore, to investigate the selective requirement of Nup358, NUP153 and Nup54 in cNLS-GFP import, they did RNAi against Nup358, NUP153 and Nup54 and found reduced level of cNLS-GFP in nucleus but no defect in GFP-NES in cytosol. They further hypothesized that the import phenotypic defect might be indirectly/ secondary due to structural defect in the NPC caused by silencing of the nucleoporins. In order to investigate the NPC integrity, they stained dsRNA treated cells with monoclonal antibody (mAb414) and a group of antibodies against NPC components. Nup214 and Nup88 were found at cytosolic face, gp210 at the central core of NPC and TPR in the nuclear basket acting as epitope. They found considerable decrease in mAb414, and anti TPR rim labeling in cells pretreated with dsRNA silencing against NUP153. So both the cytoplasmic and nuclear basket nucleoporins become severely affected by RNAi silencing of NUP153 mRNA. However, no functional defect in gp210 transmembrane protein was seen. This pointed towards the structural stability of gp210 in NUP153 depleted cells.

Investigations have shown that a short stretch of 126-132 amino-acid sequence of Simian virus 40's large T (SV-40) behaves like nuclear localization signals/ Karyophillic signal. Attachment of SV-40's large T to NPC allows as big as gold particle to enter in to the nucleus via with the intervention of Ran protein. Ran is a monomeric protein of about 24-kDa and has an intrinsic GTPase activity which plays an important role in clamping directionality on transport of biomolecules via NPC. Ran is having a core guanine binding domain (G- domain) including a p-loop, switch 1 and 2 and a 40 amino acid C-terminal which includes an acidic tail (DEDDDL).

Ran proteins normally shuttle between the nucleus and cytosol by attaching with either GTP or GDP. GTP bound Ran (Ran-GTP) is considered as active form of it, while the GDP bound Ran-GDP is the in-active form.

These two alternate states of Ran are regulated by two classes of proteins which subsequently promote the formation of either Ran-GDP or Ran-GTP forms. Ran-GAP (GTPase activating protein) and RanBPs (Ran binding proteins: RanBP1 and RanBP2) increase the intrinsic GTPase activity of Ran which subsequently leads to the formation of Ran-GDP. Ran-GEF (guanine-nucleotide exchange factor) also had known as RCC1 which increases the formation of Ran-GTP. An orchestrated cycling of active Ran-GTP and Ran-GDP between the two compartments of the cell enables the formation of Ran gradient. During this process, Ran-GEF (RCC1) is imported in to the nucleus which subsequently binds to the chromatin and makes Ran-GTP predominant in nucleus and Ran-GAP/ RanBPs in cytosol during interphase of the cell cycle. Ran has been considered as positional marker for chromatin because of its attachment with heterochromatin region. In association with RCC1, Ran leads to the formation and maintenance of the nuclear compartment. Ran acts in a number of ways to prepare the cell and assemble various structures required for the maintenance of DNA which include the nuclear envelope, spindle and the interphase nucleus (Scott *et al*., 2001).

Ran has been implicated to be up-regulated in various cancers like in prostate, breast, colon, kidney, ovarian and sarcoma. A significant up-regulation of Ran protein leads to the onset of tumorigenesis. Studies have revealed that an abnormality in Ran gradient regulating enzyme leads to the onset of breast cancer during oxidative stress.

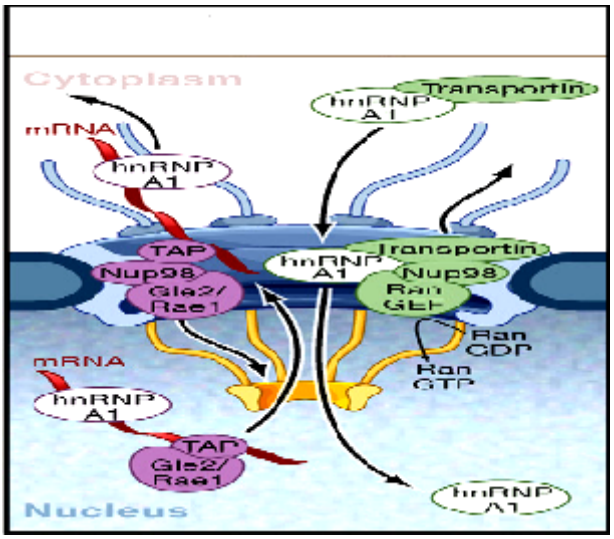

Fig 2: Ran-GTP mediated import and export of cargo-receptor complex.
(Adopted from **Cell** 2006, **Vol:** 125; 1041-1053)

1.3 NPC and its association with epigenetic control on gene expression:

Initiation process of transcription in eukaryotes plays a pivotal role in regulation of gene expression. Each gene is having its specific product which can be transcribed with different efficiency and in accordance with the need of the cell. Similarly, epigenetic control of gene expression in correlation with NPC has also been seen to play an important role in gene expression. The chromatin within the nucleus is not uniformly distributed but is organized in domains and this has a direct role to control the expression of genes located in these domains. Among the different domains of the chromatin, the peripheral domains are best characterized. The association of peripheral domains of the chromatin with the nuclear envelope via NPCs has been implicated in transcriptional repression (Kohler *et al*., 2010).

Initial evidence for the above phenomenon emerged from studies of the chromatin boundaries and the quick reactivation of inducible genes of Yeast e.g. inositol-1-phosphate-synthase (IPS) and galactokinase (GK) attain their positions throughout the nucleosol when repressed but seem to be recruited close to the individual NPCs on activation. Thus, a basket-associated supra-molecular protein network would anchor a subset of active genes like IPS and GK to the NPC, allowing the organization of an optimal microenvironment for the modulation of gene expression (Caterina *et al*., 2010).

1.4 Age dependent oxidation of NPCs and its role in aging:

NPCs breaching the nuclear envelope act as gatekeepers between the two compartments of the cell that aid in the transport and regulate the movement of biomolecules in and out of the nucleus. During the mitosis of the cell cycle, NPCs are dissembled and get distributed in the cytoplasm along with importin receptors. Subsequently, they are again incorporated in the newly formed nuclei by an orderly orchestered event of phosphorylation and dephosphorylation during the cell cycle. Recent investigation has revealed that scaffold nucleoporins e.g. NUP160, NUP107, once incorporated in NPCs are never synthesized again in fully differentiated cells/ post mitotic cells, whereas there is considerable expression of peripheral nucleoporins e.g. NUP153. Scaffold nucleoporins retain their lifelong stability within the NPC by shielding themselves inside the peripheral nucleoporins, so that they should be least accessible to proteolytic/ oxidative damage.

Studies have shown that age related deterioration of NPCs, more particularly the scaffold nucleoporins paves the way for increased cell permeability and leakiness of cytosolic proteins in to the nucleus. Leakiness in old cells has been shown due to oxidative damage to NPCs like NUP93 (Maximiliano *et al*., 2009).

1.5 Role of NPCs in the import of viral genome:

Many viruses having DNA as genetic material after getting an access to the cytosol of host cell pass through the NPCs to gain access to the nucleus in order to incorporate their genes into the host cell or remaining in the nucleus as an autonomous entity by subverting the host's replication machinery. NPCs having the small diameter create a major obstacle on their way to the nucleus. Small sized viruses easily gain an access to the nucleus e.g. hepatitis-B-virus (Dane particle) and parvovirus (MVM). The phosphorylations of the caspid of hepatitis-B-virus during maturity are actually their NLSs that serve as the binding site for importin α and importin β.

Within the nuclear basket upon interaction with importin β and NUP153, the importin β- hepatitis-B-virus interaction is dissociated by Ran-GTP and at the same time, the viral caspid attaches to the other Nups much strongly than with the importin- β. Subsequently, the mature caspid become disassembled and viral DNA finds its way to the nucleus (Schmitz *et al*., 2010).

SV40, a polyomavirus releases its subviral particle while travelling through the endoplasmic reticulum. This causes an exposure of some caspid protein NLSs and consequently these NLSs interact with importin- α and importin- β which direct them to the nucleus (Puntener *et al*., 2009). RNA based viruses like polioviruses, rhinoviruses and rhabdoviruses have a peculiar property to replicate in the cytosol of host cell. These viruses inhibit the active import of biomolecules by the proteolytic degradation of NUP153, NUP62 and NUP98 which subsequently decreases the host cell's immune response to elicit against the virus (Gustin *et al.,* 2002). Influenza virus, an Orthomyxovirus that causes a respiratory system disorder has a negative stranded RNA as the genetic material and a nucleoprotein (NP), together called as the viral ribonuclear proteins (VRNPs). These viruses have the ability to lose their capsid in the host cell's cytoplasm and consequently their RNA genome is reverse-transcribed into a complementary DNA (cDNA) in the cytoplasm.

This cDNA, along with cellular and viral protein factors forms a supra-molecular assembly termed as the pre-integration complex (PIC) which is able to mediate the cDNA nuclear import by means of different Nups e.g. NUP98, NUP358, NUP153 and RanBP2 (Woodward *et al*., 2009).

1.6 NPC and its association with nervous system disorders in humans:

Proteosomal degeneration of RanBP2 (Nup358) and the mutated version of Parkin protein having E3 ubiquitin ligase activity give rise to onset of specific neuropathies such as Parkinson's disease (Aslanukov *et al.,* 2006). Investigation of NPC proteins and its association with the Alzheimer's disease (a fatal brain disorder) reveals that a nuclear irregularity occurs in the NPC and Tau proteins (usually in association with neurofibrillary tangles). In addition, a cytoplasmic accumulation of NTF2 in hippocampal neurons (with or without tangles) is observed in Alzheimer's disease, indicating the in harmony of the transport. Also, disruptions in the distribution pattern of some karyopherins like importin-α1 are also reported in Alzheimer's disease (Lee *et al.,* 2006).

2.1 NUP153, an overview:

NPCs are mainly composed of three tiers of rings and protruding filaments on either side of the membrane which form the basket like structure towards the nuclear side. There are 8 short filaments of 3-6 nm in diameter towards the cytosolic side which are joined at the distal ring located approximately 100 nm from the mid-plane of the nuclear envelope (Fig 3). The numerous proteins of this complex are NUP153, NUP50, and NUP358 etc. NUP153 is the peripheral and mobile component of the NPCs. It is a 153 kDa glyco-protein composed of 1476 amino acids and its corresponding cDNA is of about 4.4 kb length. In association with other nucleoporin proteins, it forms a stable 3D supra-molecular assembly within the bilayer nuclear membrane. NUP153 has been found to occur peripherally towards the nucleoplasmic side of nuclear membrane. At higher resolution, using electron microscope studies, it has been seen to form part of distal ring of nuclear basket structure. Further studies have shown that the different domains of NUP153 are located in different parts of the NPC.

NUP153 is a highly mobile nucleoporins that is associated with NPC for a short span of time (in order of seconds) and is attributed with the character of dynamic nature. However, the second population of Nups is less dynamic in nature and stays at the NPC for up to 13 minutes of time interval (Rabut *et al*., 2004).

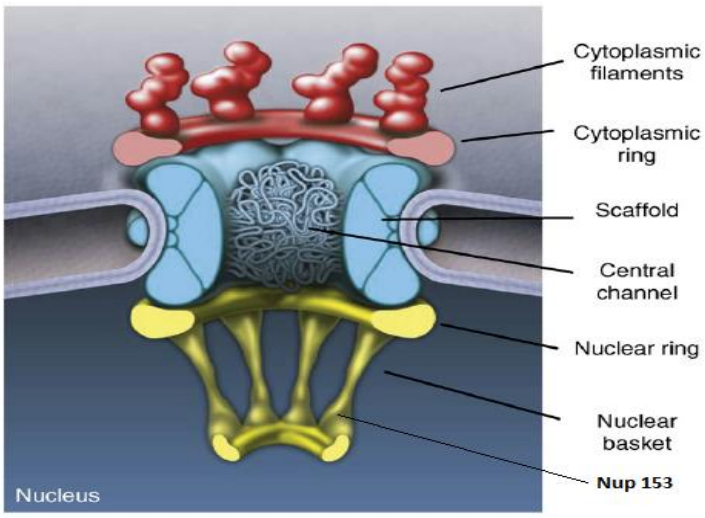

Fig 3: Nucleoporin (NUP153) located at distal region of nuclear pore towards the nucleus. (Adopted from **Cell**, 2008; **Vol** 18, 456-466)

Structure of NUP153:

NUP153 protein has three distinct domains. Each domain of NUP153 is attributed with specific functions. These are described in detail as below:

N- Terminal domain of NUP153:

The N terminal domain of NUP153 comprises of pore targeting signal sequence of the M9 domain class which is the prime pre-requisite to target NUP153 protein to NPC (Sara Nakielny *et al*., 1999). Recently, a novel RNA binding domain (RBD) was mapped within the N-terminal region of NUP153. However, the determinants of RNA association were not fully characterized (Jennifer *et al*., 2004).

Localization through HA-tagged NUP153 fragments in BHK cells revealed two overlapping segments that are involved in NUP153 localization. One segment called the nuclear envelope targeting cassette (NETC), comprising 2–144 amino-acid residues, contains a predicted amphipathic helix which directs NUP153 into proximity with the inner membrane of the nuclear envelope. Another segment, the nuclear pore associating region (NPAR) is sufficient enough to direct incorporation of NUP153 into NPCs.

Central region of NUP153:

The central domain of NUP153 contains four zinc finger motifs, constituting a novel Ran-GDP binding domain (RBD). This domain has also been shown to interact with homopolymeric RNA. This central region of NUP153 is of prime importance as it imposes directionality on the nucleo-cyoplasmic trafficking of bio-molecules. NUP153 has been shown to increase impact probability of Ran-GTP and the receptor-cargo complex dissociation in the nucleus. The NMR study of the second zinc finger of NUP153 reveals LVA motif to be important for Ran binding (Higa *et al*., 2007).

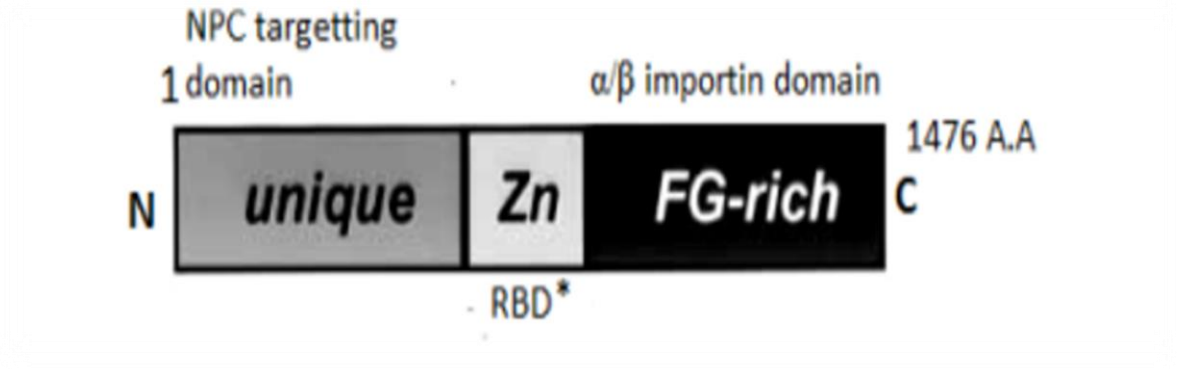

Fig 4: Picture showing various domains of Nup153 protein.
* RNA binding domain

C-terminal region of NUP153:

The C terminal region of NUP153 contains approximately thirty irregular spaced FXFG repeats (Phe (F)-any amino-acid (X)-Phe (F)-Gly (G). Different classes of FG-containing repeats are found in many nucleoporins. NUP153 domains rich in this motif share the ability to interact with import and export transport receptors. Recently, it has been reported that the FG domains of NUP153 form a brush-like structure in in-vitro condition with entropic repulsion properties (Lim *et al*., 2006).

Bio-molecules that travel through the NPC by binding to FG repeats have significantly higher transport rate than those without attachment to FG repeats, given their similar size and conformation. The weak intermolecular hydrophobic interactions among the FG repeats gives rise to a selective permeability barrier that stops the free diffusion of variety of molecules through the NPC. The weak bi-molecular hydrophobic interactions between the FG repeats containing nucleoporins and transport receptors break the zip like appearance of the tunnel of NPC and allow the passage of bio-molecules across the NPC.

2.2 Role of NUP153 in nuclear membrane breakdown:

Nucleoporins residing in intimate contact with the nuclear envelope are precisely positioned to help execute the nuclear envelope breakdown. Investigations have revealed that NUP153 in collaboration with COP1 (Coatomer Protein 1) complex aids in the execution of nuclear envelope break down.

COP1 has already been extensively studied in context with vesicle budding during the transport of immature peptides with Golgi and transport from endoplasmic reticulum to Golgi apparatus (Jin Liu *et al*., 2003).

An experiment was performed by taking Xenopus cell free extract to form synthetic nuclei around sperm chromatin. Subsequently, cycloheximide, a eukaryotic translational inhibitor was introduced to prevent the expression of cyclins and thereby arresting this cell free extract in the interphase of cell cycle. Later on, a stable recombinant form of cyclin B was added to execute the synchronization and the mitotic event. Results showed that among the three different recombinant constructs of NUP153 (GST-NUP153, GST-Z, GST-N) expressed in Xenopus cell free extract, only GST-Z recombinant protein formed (fragment containing the central zinc finger domain of NUP153) showed marked inhibition of the nuclear envelope break down which was subsequently confirmed by staining the nucleus with DAP1 stain to check the nuclear membrane integrity.

In an another attempt to see the interaction between recombinant zinc finger domain (GST-Z) of NUP153 and various Xenopus proteins, recombinant zinc finger protein was immobilized on sepharose beads which were then subsequently incubated with Xenopus egg extracts. Mass spectroscopic analysis revealed that three members of the COP1 coatomer complex members (β, β` and α) specifically interact with zinc finger domain of NUP153. More specifically, when antibodies were generated against β-coatomer and then directed against it, they prevented nuclear envelope breakdown, suggesting that COP1 plays an important role in the nuclear envelope disassembly.

2.3 Role of NUP153 in DNA Damage Response (DDR):

NUP153 plays an important role in the recruitment of p53BP1 (p53 binding protein) to the nucleus in response to cellular DNA Damage response (DDR). Normally when a cell faces DNA double stranded breaks (DSBs), proximal DDR kinases (ATM, ATR kinases) play their prime role by recruiting an array of repair factors to DNA damage foci including p53BP1. p53BP1 plays an important role from telomere maintenance to replication stress responses by stalling the under-replicated genomic loci in G1 phase of the cell cycle.

NUP153 as such has no role in the regulation of p53BP1 expression but plays a vital role in redistribution of p53BP1 during DDR response by recruiting the p53BP1 at the site of foci formation. Despite the role of NUP153 in the nuclear accumulation of p53BP1 in response to IR induced DDR, NUP153 knockdown did not hamper the global transport of other repair factors to the nucleus. In the NUP153 deficient cells irradiated with a dose of 2GY (IR), MDC1 nuclear DSB foci formation was followed. The number of MDC1 foci formed at DSB was found out to be the same before and after the IR treatment.

NUP153- p53BP1 bi-molecular cross talk was investigated by using siRNA against NUP153 in HeLa cells and subsequently observing up to 8 hours after IR treatment. Investigations confirmed that although total expression of 53BP1 remained unchanged in HeLa cells but p53BP1 localization partially redistributed to the cytoplasm largely after NUP153 knockdown as was seen by immunofluorescence microscopy. The partial distribution of p53BP1 between the cytosolic and nuclear fractions was also seen by immunoblots which reflected the consequence of variable impact on 53BP1 subcellular localization in NUP153 depleted cells, in otherwise unperturbed cells. NUP153 knockdown slightly affected the nuclear localization of BRCA1 and had no effects on DDR factors e.g. MDC1, NBS1and Rad51. Finally, to confirm the specificity of this observed phenotype, p53BP1 nuclear accumulation and formation of spontaneous and IR-induced p53BP1 foci formation in these HeLa cells were rescued by the expression of siRNA-resistant GFP-NUP153 construct (Moudry *et al*., 2012).

2.4 NUP153 and its association with the regulation of spindle check point:

NUP153 has been found to be important in cell cycle regulation by localizing the spindle assembly checkpoint (SAC) protein Mad1 to NPCs in the interphase stage of cell cycle. The SAC executes its work by preventing the aneuploidy and chromosomal mis-segregation and delaying the metaphase-anaphase transition until all chromosomes are properly attached to the mitotic spindle and aligned at the metaphase.

Mad1 and Mad2, the two SAC proteins have been found to be located at NPCs in the interphase cells. Mad2 is thought to play an important role for mitotic checkpoint because of its inhibitory effect on the anaphase promoting complex/cyclosome

(APC/C). The binding of Mad2 to Mad1 and subsequently with Cdc20, a co-factor of APC/C, is thought to be crucial for Mad2 function. Loss of the orderly orchestrated interaction between these proteins ensues in impaired function of SAC and failure of cytokinesis. The role of Mad1 during metaphase/anaphase transition on the other hand appears regulatory as the depletion of Mad1 results in SAC deficiency without significantly altering the duration of mitosis. The interaction of NUP153 with Mad1 is of prime importance in the regulation of the spindle checkpoint during the cell cycle. Overexpression of human NUP153 in HeLa cells gives rise to the appearance of multinucleated cells and induces the formation of multi-polar spindles (King *et al*., 2008).

NUP153 is important for regulating the localization of Mad1 in interphase of cell cycle Results have shown that the depletion of NUP153 by using RNA interference ensued in the decrease of Mad1 at nuclear pore during interface of the cell cycle which causes tremendous delay in the dissociation of Mad1 from kinetochores in metaphase and an increase in number of unresolved mid-bodies. In order to determine which domain of NUP153 interacts with Mad1, solution-binding assays were performed. Recombinant NUP153 domains in frame with GST were attached to glutathione sepharose beads and incubated with in-vitro translated S^{35} labeled Mad1 protein. Mad1 was found to interact with GST-NUP153 with the N-terminal domain of NUP153 and a truncation of the N-terminal domain comprising the first 339 residues of NUP153 but not with GST alone. Therefore, the interaction of NUP153 with Mad1 was found to be mediated by NUP153's NPAR (Nuclear Pore Associating Region).

A functional SAC is a supra-molecular assembly of Mad1, Mad2 and Cdc 20. Mad1 is hyperphosphorylated when bound to Mad2 in mitosis and phosphorylation of Mad1 is critical for SAC function. In order to investigate whether NUP153 effects Mad1 phosphorylation, co-immunoprecipitation experiments of cells after nocodazole treatment were performed.

Mad1 protein was immunoprecipitated from lysates of nocodazole arrested HeLa cells that were either transfected with GFP-NUP153 or NUP153 siRNAs, using a monoclonal Mad1 antibody. Mad1 phosphorylation was assessed by western blotting using an antibody that recognizes a phosphorylated serine /threonine/or tyrosine residue (-pSTY).

Indeed, the -pSTY antibody detected phosphorylated Mad1 in control cells, whereas the levels of phosphorylated Mad1 were reduced by ~10% in GFP-NUP153 transfected cells but not in NUP153-depleted cells.. The amount of Mad2 that co-precipitated with Mad1 was not affected in cells expressing GFP-NUP153 or NUP153-depleted cells. However, the reduced levels of NUP153 on the contrary do not impair Mad1's phosphorylation status, leaving the SAC functional for cell cycle regulation. Together these data suggest that an increased level of NUP153 derails the SAC function by impairing Mad1 phosphorylation without affecting its association with Mad2 and thereby affecting the cell cycle regulation (Lussi *et al*., 2010).

2.5 Role of Nups in transcriptional regulation:

Using chromatin immunoprecipitation (ChIP) in combination with microarray hybridization, results have shown that NUP153 and Megator (Mtor) bind to 25% of the genome in continuous domains extending 10kb to 500kb. The nucleoporins associated regions (NARs) are enriched by markers for active transcription, including RNA polymerase II and histone H4K16 acetylation. Studies have shown that Knock-down of NUP153 alters the expression of approximately 5,700 genes with a marked down-regulatory effect within NARs. It has also been found that nucleoporins play a central role in coordinating dosage compensation, an organism-wide process involving the doubling of expression of the male expression of the male X chromosome. NAR are dominant on the male X chromosome and occupies 75% of this chromosome. Further, NUP153 depletion vanishes the normal function of the male-specific dosage compensation complex. Using extensive three dimensional imaging, results have demonstrated that NARs contribute to control of gene expression irrespective of their sub-nuclear localization. Therefore, NAR binding is used for chromosomal organization that enables the gene expression control (Vaquerizas *et al*., 2010).

2.6 NUP153 and its role in onset of laminopathies:

The nuclear envelope and the underlying nuclear lamins are of crucial importance for maintaining the integrity of the nucleus as well as for positioning of NPC. The NUP153 has been previously reported to bind to the B-type lamins, but there is as such no exquisite specificity for binding with NUP153. The NUP153 exhibits multiple binding sites for A- and B- type lamins.

Using GST pull down assays, it was found that the N & C-terminal domain of NUP153 associate with the Ig folds of B- and A- lamins. It was found that if lamins are mutated, they were unable to bind to the NUP153. Thus, NUP153 plays a vital role in lamin associated diseases collectively called Laminopathies (Al- Haboubi *et al*., 2011).

2.7 Role of NUP153 in tumorigenesis:

NUP153, besides being the indispensible dynamic component of NPCs maintains the nuclear envelope architecture. Recent investigations have revealed that NUP153 plays an important role of tumor cell migration in metastatic breast carcinoma (Lixin *et al*., 2010). NUP153 depletion in human breast carcinoma cell line (MDA-231) affects the polarity and directional cell migration which is basically the fundamental property of tumor cells to invade other regions. NUP153 has been earlier implicated in cytoskeletal rearrangement. Keeping this in mind, RNAi silencing of NUP153 in MDA231 cell lines was done. As a result, the expression of NUP153 was significantly reduced and large rearrangement of microtubule cytoskeleton and actin was found. Since microtubules and actin play an important role in cell motility, migration response of NUP153-RNAi treated MDA231 breast carcinoma cell line was investigated. It was found that wound closure occurred at significantly slower rate in NUP153-RNAi treated MDA231 breast carcinoma cell lines than in the untransfected ones.

Similarly, cell orientation was also checked in NUP153-RNAi treated MDA231 breast carcinoma cell line by locating Golgi-apparatus and MTOC (Microtubule Organizing Centre) and it was found that they were unable to change their position towards the wound. In mock transfected MDA231 cell lines, Golgi-apparatus and MTOC were found to be oriented towards the wound.

2.8 Role of NUP153 in apoptosis:

NUP153 is targeted for disruption during apoptosis. This attack on nuclear pore components appears to take place in an orderly orchestrated event which eventually disables the structural conformation and transport role of the nuclear pores. NUP153 cleavage corresponds to the pore clustering and in association with cytoplasmic alterations may contribute to the disruption of nuclear morphology. Indeed, apoptosis is experimentally induced in some experiments by extensive treatment of transcriptional inhibitors like actinomycin-D, which is also well known to stall NUP153 mobility.

It is not yet clear if this is caspase dependent cleavage or not. It is noteworthy to mention here that the putative NUP153 caspase-3 cleavage site occurs within the RNA binding domain of its N-terminal region. Possibly the lack of RNA cargo under condition of transcriptional inhibition, leaves the NUP153 prone to caspase cleavage. So, NUP153 plays an important role in executing the process of apoptosis by itself losing its position towards the distal region of NPC (Jennifer *et al*., 2005).

3.1. Materials

3.1.1. Chemicals used

Acetic-acid ($C_2H_4O_2$), Agar, Agarose, Ampicillin, Bromophenol-blue (BPB)**,** Bovine serum albumin (BSA)**,** Calcium chloride ($CaCl_2$), Ethanol (C_2H_5OH), Ethidium bromide (EtBr), Ethylene diamine tetra-acetate (EDTA), Glycerol ($C_3H_8O_3$), Isopropanol, Pottasium hydroxide (KOH), LB Agar, LB Broth, Piperazine sulphanilic acid (PIPES), Restriction-enzymes, Tris acetate ethylene diamine tetra acetate (TAE), Tris EDTA (TE), Ribonuclease (RNAse), Sodium chloride (NaCl) and Sodium hydroxide (NaOH).

(*All the chemicals used were of Standard Molecular Biology Grade and were obtained from reliable sources viz Himedia, Fisher Scientific, Invitrogen, Merck, and Sigma- Aldrich etc).*

3.1.2. Baterial strains used for transformation purpose

E. coli; XL –Blue strain (dam^-/ dcm^- genotype) and DH5-α competent cells.

3.1.3. Vectors used for cloning

pCMV-HA (3.7kb) was obtained from Addgene, USA (Cat. No: Plasmid #32530) and pcDNA3 (5.4kb) was obtained from Invitrogen. Both these vectors are mammalian expression vectors and are commonly used for transient transfection purposes.

3.1.4. Enzymes used for cloning

NotI, XhoI, HindIII, Vent polymerase and DNA ligase were purchased from New England Biolabs (NEBS) .

3.2. Kits

3.2.1. Plasmid extraction Miniprep kit, PCR purification kit was obtained from StrataPrep® and Sigma Aldrich respectively.

Sequences of primers used in PCR amplification.

Table 2: Sequences of primers used for amplification of NUP153 recombinant NUP153-pCMV-HA construct.

Primer	Direction	Sequence	Tm	Used for
Primer1 (NUP153 XhoI)	Forward	5' GCCGCCTCGAGATG CCTCGGGAGCCGG3'	89.1^{0}C	Standard PCR
Primer2 (NUP153 NotI)	Reverse	5'GCCGCGCGGCCGCTTAT TCCTGCGTCTAACAGC3'	87.2^{0}C	Standard PCR

Table 3: Sequences of primers used in amplification for NUP153 recombinant NUP153-pcDNA3.O construct.

Primer	Direction	Sequence	Tm	Used for
Primer 1 (NUP153 HindIII)	Forward	5'GCCAAGCTTTTATTT CCTGCGTCTAACAGC3'	79.8^{0}C	Standard PCR
Primer2 (NUP153 XhoI)	Reverse	5'GCCGCCTCGAGATG GCCTCGGGAGCCGG3'	80.3^0 C	Standard PCR

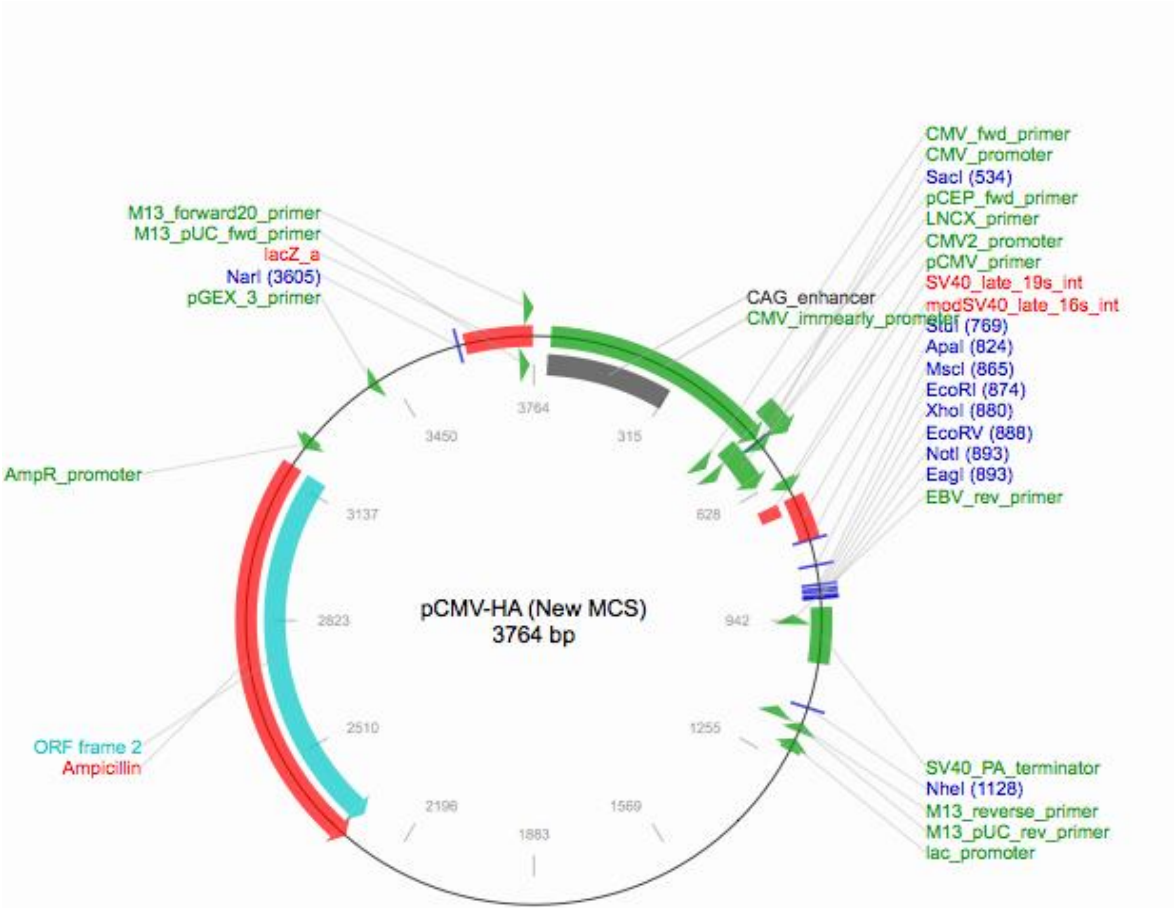

Fig 5: Map of pCMV-HA mammalian expression vector of about 3.7kb.

(Adopted from www.addgene.com).

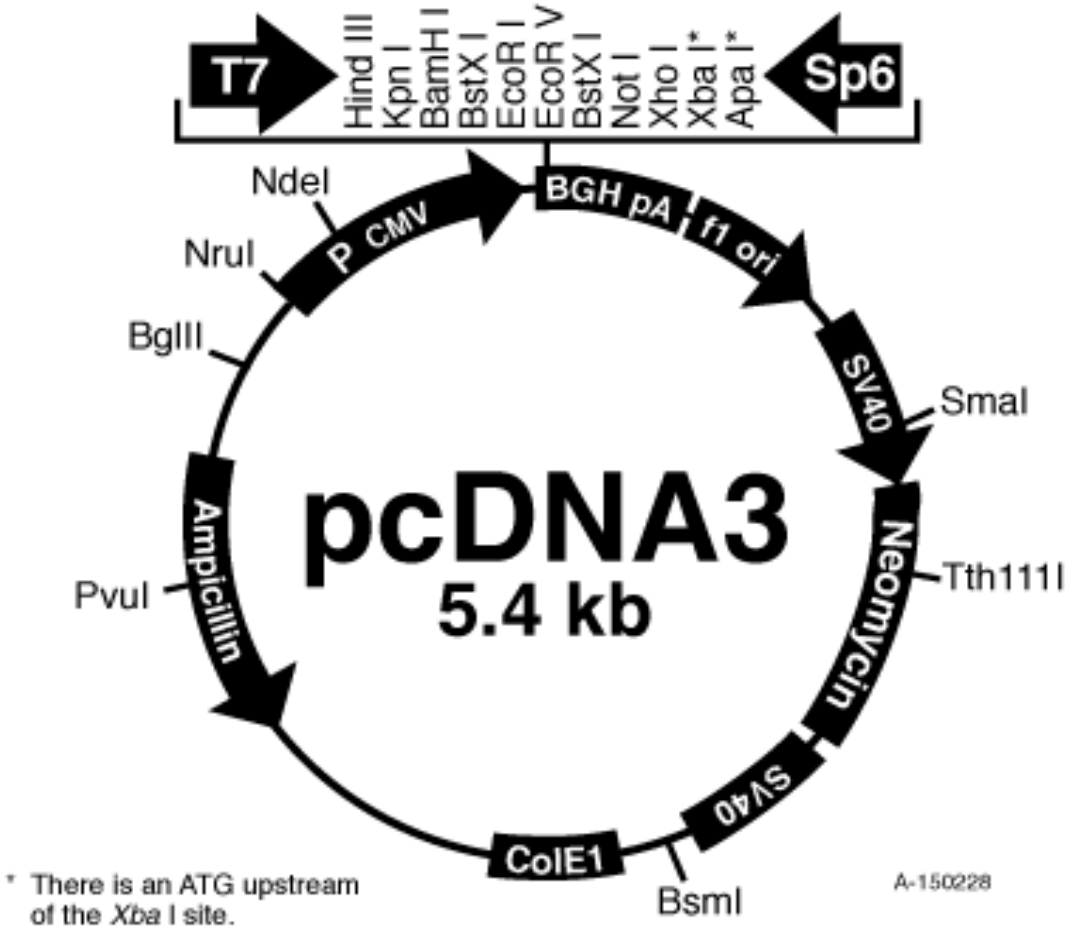

Fig 6: Map of pcDNA3.0 mammalian expression vector of about 5.4 kb.

(Adopted from *Invitrogen*)

3.3. Methods:

3.3.1. PCR amplification and reagents for PCR amplification:

Polymerase chain reaction (PCR) was used to amplify the desired cDNA of Nup153 that was already in pCMVSPORTS 6.0 obtained from ATCC, USA. Forward and reverse primers were designed to incorporate compactible restriction sites at the 5' ends of the primers. (For Primer details see above Table 2 and 3). The below mentioned text describes a typical PCR reaction and cycling program's.

PCR reaction mixture (for NUP153-PCMV-HA construct and NUP153-pcDNA3.0 Construct)

1.0 µlcDNA of pCMV-SPORTS 6.0-Nup153 (Template)

2.5 µl 5' oligo forward

2.5 µl 3' oligo reverse

2.0 µl dNTP

10 µl 10x Vent buffer

76 µl H_2O

1.0 µl Vent DNAPolymerase

Total 100µl

PCR cycling programme for (NUP153-pCMV-HA construct)

94°C 3 min (1 cycle)

94°C 1 min

67°C 30 sec

72°C 6 min (30 cycles)

72°C 10 min (1 cycle)

Hold temperature at 4°C

PCR cycling programme for (NUP153- pcDNA3.0 construct)

94°C 2 min (1 cycle)

94°C 30 sec

76°C 30 sec

72°C 6 min (30 cycles)

72°C 10 min (1 cycle)

Hold temperature at 4°C

3.3.2. Agarose gel electrophoresis and the purification of PCR product.

To check the quantity and the quality of the PCR amplified products, 5µl of the PCR product were loaded on 0.8% agarose gel containing ethidium bromide (Et-Br). After electrophoresis for about 30-40 minutes, the gel was visualized using gel-doc ultraviolet light system and photographed. The concentrations of the amplified products were approximated by comparison with the intensity of the corresponding bands in 1kb DNA ladder.

The PCR products were purified by the following method:

3.3.3. Purification of both PCR products using gel purification kit:

Gel purification of PCR amplified products were performed as per the protocol supplied by the manufacturer. Briefly, the DNA bands to be purified were excised from the agarose gel with a sterile blade. The sliced gels were weighed and collected in a 1.5ml tube. The gel slices were suspended in Solution 1 with three times the volume of the excised band/ slice and were subsequently incubated at 65^0 C for about 10-15 minutes to allow the gel to melt completely. One volume of isopropanol was then added to it. In the meantime, columns were prepared by incubating 500µl of column binding buffer solution in binding column and then centrifuged at 13000 rpm for 3 min.

The molten gel/ DNA solution was applied to the columns and centrifuged at 13000 rpm. Subsequently 750µl of ethanol wash buffer was added to the columns and centrifuged at 3000 rpm for 2 minutes.

The tubes were again centrifuged to remove the residual ethanol wash buffer. 50µl of pre heated elution buffer was introduced in each column to get the products.

3.4. Molecular cloning:

The PCR amplified fragments were cloned in mammalian expression vector pCMV-HA got from Addgene (Cat. No: Plasmid #32530) and pcDNA3.0 from Invitrogen using the following procedure:

Digestion of the amplified products were done as per the below given protocol:

- Restriction digestion of the amplified product (Nup153) with Xho1 and Not1 restriction enzymes meant for Nup153-pCMV-HA construct is given below (Table 4).

S.no	Reagents	Volume (µl)
1.	Water	1.0
2.	Amplified product/NUP 153	50.0
3.	NEB Buffer- 4	6.0
4.	XhoI	1.0
5.	NotI	1.0
6.	BSA	1.0
	Total volume	**60.0**

- Restriction digestion of the amplified product (Nup153) with Hind111 and XhoI restriction enzymes meant for Nup153-pcDNA3.0 construct is given below (Table 5).

S.no	Reagent	Volume (µl)
1.	Water	1.0
2.	Amplified product NUP153	50.0
3.	NEB Buffer- 4	6.0
4.	HindIII	1.0
5.	XhoI	1.0
6.	BSA	1.0
	Total volume	**60.0**

- Restriction digestion of the vector DNA (pCMV-HA) with XhoI and NotI restriction enzymes meant for Nup153-pCMV-HA construct is given below (Table 6).

S.no	Reagent	Volume (µl)
1.	Water	2.5
2.	Vector DNA (pCMV-HA)	40
3.	NEBS Buffer 4	5
4.	XhoI	1
5.	NotI	1
6.	BSA	0.5
	Total volume	**50 µl**

- Restriction digestion of the vector DNA (pcDNA3.0) with HindIII nd XhoI restriction enzymes meant for Nup153-pcDNA3.0 construct is given below (Table 7).

S.no	Reagent	Volume (µl)
1.	Water	30
2.	Vector-DNA(pcDNA3.0)	10
3.	NEB Buffer 4	5
4.	HindIII	2
5.	XhoI	2
6.	BSA	1
	Total volume	**50 µl**

The reactions were incubated at 37ºC for 10-12 hrs (overnight). Digested products were purified by Sigma Aldrich gel Purification kit as described previously.

3.5. Ligation reactions:

The ligation reaction of Nup153 PCR product and pCMV-HA meant for Nup153-pCMV-HA construct was carried out as per the below given protocol (Table 8):

S.No	Reagents	Control reaction Control (µl)	Ligation mixture (1) (µl)	Ligation mixture (2) (µl)	Ligation Mixture (3) (µl)
1.	Vector (pCMV-HA)	2.0	2.0	2.0	2.0
2.	Insert (NUP153)	0.0	4.0	6.0	8.0
3.	10X ligase buffer	2.0	2.0	2.0	2.0
4.	T4 DNA ligase	1.0	1.0	1.0	1.0
5.	Water	15.0	11.0	9.0	7.0
	Total volume	**20 µl**	**20 µl**	**20 µl**	**20 µl**

The ligation reaction protocol of purified Nup153 PCR product and pcDNA3.0 meant for Nup153-pcDNA3 construct was carried out as per the below given protocol (Table 9):

S.NO	Reagents	Control reaction Control (µl)	Ligation mixture (µl) (1)	Ligation mixture (µl) (2)	Ligation Mixture (µl) (3)
1.	Vector (pcDNA3.0)	1.0	1	1.0	1.0
2.	Insert (NUP153)	-	1.0	2.0	3.0
3.	10X ligase buffer	2	2	2.0	2.0
4.	T4 DNA ligase	0.5	0.5	0.5	0.5
5.	Water	16.5	15.5	14.5	13.5
	Total volume	**20 µl**	**20 µl**	**20 µl**	**20 µl**

The contents mentioned above used in ligation reation were vortexed and spun down for 3-5 seconds and incubated overnight at 16^0C.

3.6. Preparation of competent cells and transformation:

3.6.1. Calcium Chloride method (Cohen et al., 1973):

This method allows the long term storage of the competent cells without losing their ability of competence to take up the foreign DNA. For the preparation of competent cells, 3ml LB broth was inoculated with XL-Blue E.coli strain (dam^-/dcm^-) and was grown overnight at 37°C with constant shaking.

500µl of the overnight culture were introduced into 50ml LB broth (1:100 dilution) in a 50ml flask and incubated for 3-4 hrs at 37°C, till the absorbance at 600 nm reached 0.5 OD. The culture was chilled on ice for about 10 minutes. Cells were harvested by centrifugation at 7,000 rpm for 15 min at 4°C.

Supernatant was discarded and the tubes were placed in an inverted position to allow the last traces of the medium to be drained out. Pellet was resuspended in approximately 10ml of 0.1M ice-chilled calcium chloride and subjected to centrifugation at 10,000 rpm. The pellet so obtained was again resuspended in 10ml of 0.1M calcium chloride solution and again subjected to centrifugation. The pellet so obtained was suspended in 2ml of calcium chloride solution (60mM of $CaCl_2$, 15% of Glycerol and 10mM of PIPES). Cell suspension was stored on ice and dispensed into 100µl aliquots in ice chilled sterile 1.5ml storage tubes and stored at -80°C until needed.

3.7. Transformation of E. Coli XL-Blue Competent cells:

Before transformation, a 50µl aliquot of competent cells was thawed on ice. The ligation reactions (about 5µl) to be transformed were then added to the competent cells and incubated on ice for 15 min and then subjected to heat shock treatment at 42^0C for 45 seconds. The cells were then incubated on ice for additional 2 minutes. Subsequently, about 1ml of LB broth without ampicillin was added to these cells and the mixture was incubated with shaking at 37^0C for 1 hour. The mixture was then spun down and the pellet was resuspended in about 200µl was then spread on LB Agar plate containing 100µg/ml ampicillin. The plates were then incubated at 37°C overnight.

3.8. Plasmid isolation using Plasmid Miniprep kit.

The bacterial colonies that grew on LB-Agar plates were used for plasmid extraction. The StrataPrep® plasmid miniprep kit was used for isolation of the plasmids following the methods described by the manufacturer. The method employs a modification of the Alkaline Lysis method. Briefly, the bacterial culture grown overnight (1.5ml) was centrifuged for 1 minute and the pellet was suspended in 100 ul of ribonuclease-containing solution (Solution 1) for 5 minutes. 100ul of solution (2) was added for 5 minutes and the mixture was inverted several times to facilitate mixing and lysis. This was followed by the addition of 125 ul of Solution (3) for 5 minutes and thorough mixing which caused the precipitation of the cellular lysate.

The precipitate was then centrifuged at 14000 rpm for about 10 minutes and the supernatant (about 300µl) was then transferred to the supplied column and centrifuged for 1 minute. The plasmid DNA binds to the fiber matrix in the column. The contaminants were washed from the column with the supplied wash buffer (750µl). The residual wash buffer were removed by additional centrifugation for 30 sec and air-dried for 1 min. The plasmid DNA was then eluted from the column with the elution buffer (50µl) and recovered in a micro centrifuge tube. The purified plasmid DNA thus obtained was then used for subsequent steps like the restriction digestion, ligation etc. Using this kit, about 200-300µg of the plasmid DNA was routinely obtained. Whenever desired, the quality of the DNA was assessed by running 3-5 µl of the plasmid on 0.8% of the agarose gel. Plasmid DNAs so obtained from plasmid mini-prep extraction were used for restriction digestion as per the protocol described above.

4.0. Results:

4.1. Assessment of the transformed pCMVSPORTS 6.0- NUP153 Plasmid DNA:

The plasmid pCMV-SPORT6.0- NUP153 was obtained from ATCC, USA. This plasmid was transformed into DH5α competent cells. About four colonies were picked up for plasmid purification, which was done by the Alkaline Lysis method. About 5µl of the plasmids were run on 0.8% agarose gel to assess the quality and quantity of the plasmids. Results showed that plasmids were successfully obtained as shown in the figure 7.

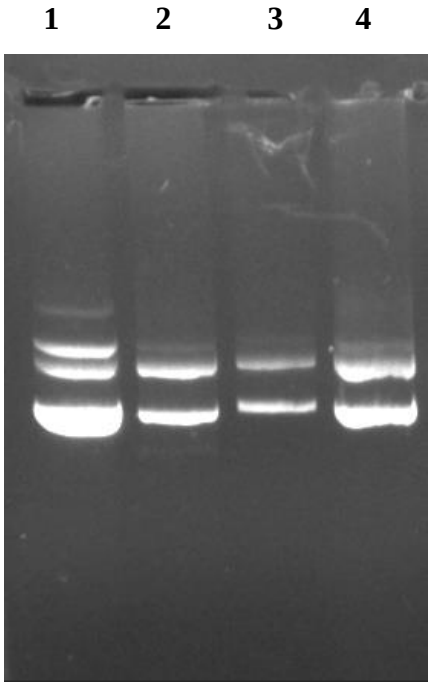

Fig: 7. Representative gel picture showing extracted plasmid in all lanes.

Primer design and cloning:

Primers were designed in such a way that forward primer (NUP153-XhoI-F) carried XhoI restriction site at its 5' end and the reverse primer (NUP153-NotI-R) carried a NotI restriction site to ensure directional cloning in pCMV-HA mammalian expression vector and facilitate the generation of HA tag in frame.

Primers were also generated to make another construct in pcDNA3.0 mammalian expression vector with forward primer (NUP153-F-HindIII) carrying HindIII restriction site and reverse primer (NUP153-R -Xho1) carrying Xho1 restriction site, as shown below:

Forward Primer for NUP153- pCMV-HA construct.

5'---**GCC**GCCTCGAGATGGCCTCGGGAGCCGG----3'

---- XhoI----

Reverse Primer for NUP153- pCMV-HA construct.

5'---**GCC**GCGCGGCCGCTTATTTCCTGCGTCTAACAGC3'

----NotI----

Forward Primer for NUP153-pcDNA3.0 construct.

5'---GCCAAGCTTTTATTTCCTGCGTCTAACAGC3'

--HindIII-

Reverse Primer for NUP153-pcDNA3.0 construct.

5'---GCCGCCTCGAGATGGCCTCGGGAGCCGG----3'

----- XhoI----

4.2. PCR amplification of NUP153:

Using pCMVSPORT 6.0-NUP153 as the template, PCR reactions (100µl each) were done using the above set of primers, using conditions as mentioned in the Materials and Methods section. The reaction mixtures were run on 0.8% agarose gels. Results showed a specific band of the desired size of 4.4kb. The PCR products were excised from the gel and subjected to the gel purification using the Sigma Aldrich gel purification kit (See figure 8).

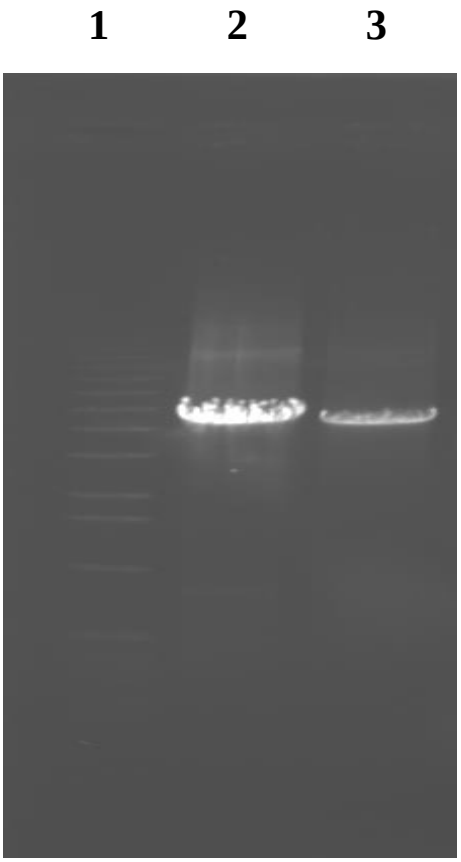

Figure: 8. Agarose gel electrophoresis showing the amplification of cDNA from pCMV-Sport 6- Nucleoporin 153 (NUP153). Lane-1 represents 1kb DNA ladder. Lane-2 & Lane-3 show amplification of 4.4kb fragments generated by two different sets of primers as mentioned above.

4.3. Restriction Digestion:

For cloning the PCR products in the mammalian expression vectors, the next step was to carry out the digestion of PCR products as well as the vectors. This was done by setting up the digestion reactions of the purified PCR products (in 50µl total volume) as well as the vectors (10µg each). The reactions were incubated for overnight and subsequently run on the 0.8% agarose gel to confirm complete digestion, as could be assessed by the appearance of a single linear band of the vectors (Figures 9 and 10).

The mammalian expression vector pCMV-HA (3.7Kb) was digested with XhoI and NotI restriction enzymes. Similarly, mammalian expression vector pcDNA3.0 was digested with HindIII and XhoI restriction enzymes.

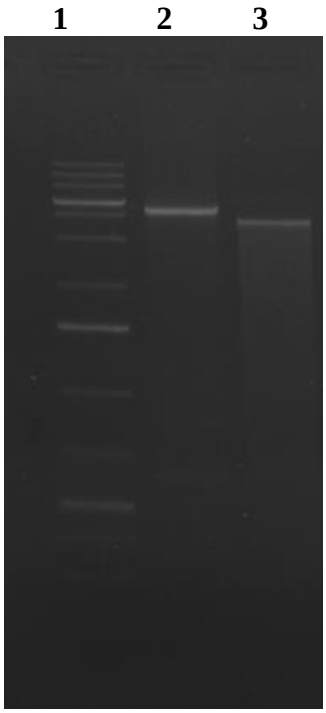

Fig: 9

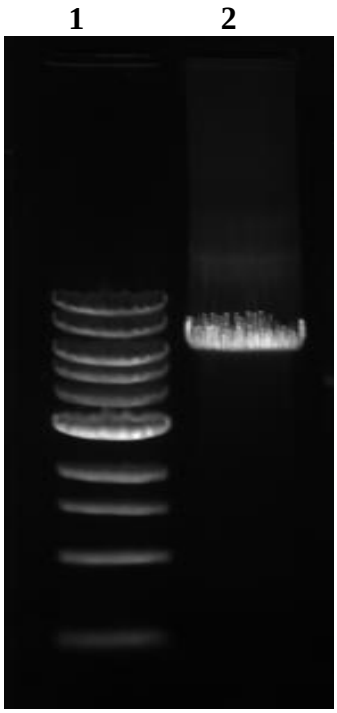

Fig: 10

Fig: 9. Lane 1 shows 1kb DNA ladder, lane 2 shows amplified NUP153 band after digestion and PCR purification, lane 3 shows digested pCMV-HA vector band after gel purification.

Fig:10. Lane 1 shows 1kb DNA ladder and lane 2 shows digested pcDNA3.0 (vector) band after gel purification.

Ligation reaction:

Three separate ligation reactions using constant amount of the vector but variable amounts of the inserts (typically 1:1, 1:3 and 1:5 ratio's of the vector: insert) were setup for both the constructs (NUP153-pCMV-HA and NUP153-pcDNA3.0) (protocol given in Materials and Methods section).

In addition, only the vector (with no inserts) ligation reactions were used as the controls. About 5µl of the ligation reactions were used for transformation of the host in XL- blue competent cells using standard protocol. The transformed mixtures were grown in LB for about an hour and later plated on LB-Agar plates containing ampicillin. For the first construct (NUP153- pCMVHA), we got two colonies on control plate that contained only the vector (pCMV-HA).

In contrast the plate with ligation mixture (1) had nine colonies, the one with ligation mixture (2) contained thirteen colonies and the one containing ligation mixture (3) had seventeen colonies after overnight growth on LB-Agar ampicillin plate.

Similar strategy was used for the cloning of NUP153 in pCDNA3.0 vector also. After ligation and transformation, followed by incubation on LB-Agar plates containing ampicillin, we got no colonies on control plate, five colonies for ligation mixture (1), nine colonies for ligation mixture (2), and thirteen colonies for ligation mixture (3). Subsequently, we randomly picked up the colonies from each LB-Agar plates and subjected them to plasmid extraction. Plasmid presence and purity were confirmed by 0.8% agarose gel electrophoresis.

The recombinant NUP153-pCMVHA construct so obtained was subsequently digested with XhoI and NotI to confirm the successful cloning of NUP153 (Figure 11). Similarly, the recombinant NUP153-pcDNA3.0 construct so obtained was also digested with HindIII and XhoI respectively to confirm the presence of the NUP153 in pcDNA3 (Figure 12).

4.4. Result of restriction digestion of recombinant pCMV-HA- NUP153 construct:

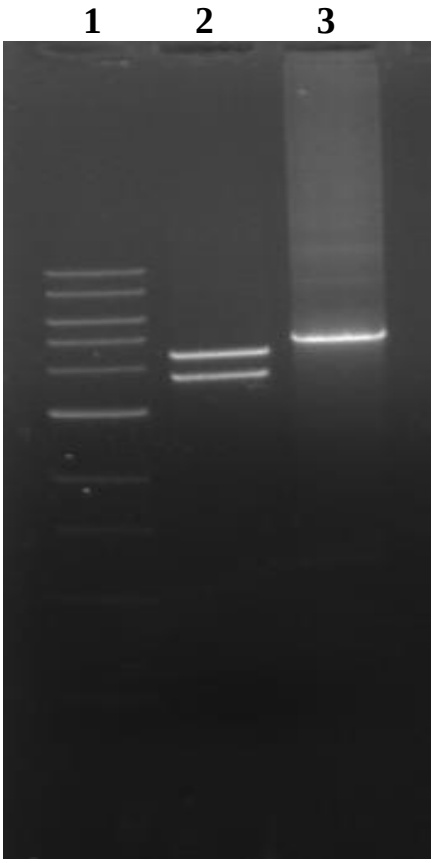

Figure: 11. Restriction digestion of the PCMV-HA -NUP153 recombinant plasmid. Lane 1 shows 1kb DNA ladder; lane 2 represents the recombinant plasmid digested with XhoI and NotI, and lane 3 represents PCR amplification of the HA-NUP153 using primers between pCMV promoter of pCMV-HA vector and the 3'-terminal end of NUP153.

4.5. Restriction digestion of the recombinant pcDNA3.0 -NUP153 construct:

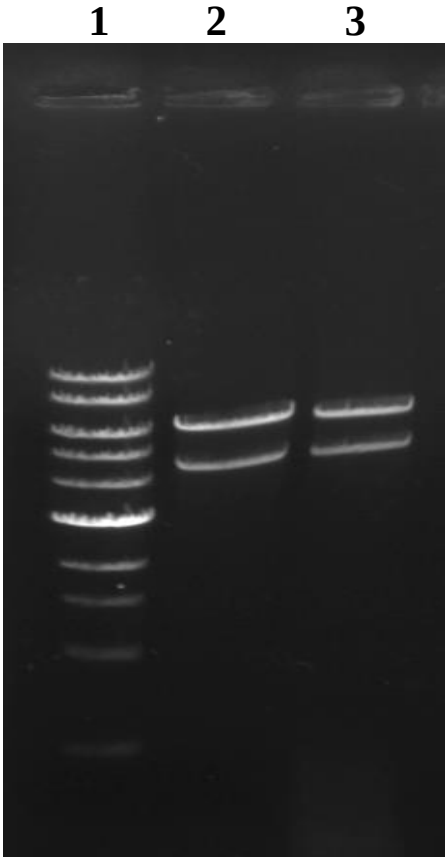

Figure: 12. Restriction digestion of the pcDNA3 -NUP153 recombinant plasmid. Lane 1 shows 1kb DNA ladder; lanes 2 & 3 represent digested recombinant plasmid digested with HindIII and XhoI.

5.0. Discussion and conclusion

5.1. Discussion

NUP153, the prime, indispensible and dynamic component of NPCs maintains the nuclear envelope architecture and aids in transport of different bio-metabolites across the bilayer nuclear membrane. NUP153 because of its remarkable property of dynamicity associates with NPCs for a very short span of time (in order of seconds) as compared to other nucleoporins which associate with NPCs for about minutes or so. NUP153 has been attributed with numerous functions so far. NUP153 have already been shown to interact with different proteins to execute its assigned function. Polyoma small T antigen has been shown previously to interact with protein phosphatase 2A (PP2A) (Pallas et al., 1999). PP2A is a multifunctional cellular phosphatase that consists of a structural (A) subunit, a catalytic subunit (C) and numerous regulatory subunits (B). Regulatory subunits in turn have numerous isoforms, which makes it possible to form more than 100 isoforms of PP2A. PP2A is having an important role in different cellular processes including apoptosis, cell cycle and cellular transformation. Polyoma small T is one of the three proteins that are synthesized by alternative splicing mechanism of the viral mRNA transcript. Small T protein (ST) is of about 195 amino-acids long which codes for about 20 kDa ST protein and has been reported to bind with PP2A. Normally, PP2A is equally distributed in nucleus and cytosol of normal eukaryotic cells. However, immunofluorescence studies have revealed that it occupies the nuclear localization mostly in ST expressing cells (Unpublished data). So in the wake of above statements, we could probably think of NUP153 as a key player which might aid in the transport of ST and PP2A concomitantly or separately.

Our prospective study of the NUP153 could be undertaken to understand whether NUP153 has any role in the transport of PP2A and ST. It may be quite possible that ST interact with NUP153 directly or indirectly via with the mediation/ intervention of PP2A or ST. Based on the above assumption, we could possibly assume three models:

- NUP153 interacts with PP2A and ST directly and independently.
- NUP153 interacts with PP2A indirectly by binding with ST of ST-PP2A complex.

- NUP153 interacts with ST indirectly by interacting with PP2A of PP2A- ST complex.

In the future studies, we would like to find out whether one of these models is true, and what implications the interaction of these components would have on cell functioning.

5.2. Conclusion

In context with the large array of data generated from last two decades about the NUP153, we successfully cloned our gene of interest in two mammalian expression vectors viz pcDNA3.0 and pCMV-HA. We generated these two constructs to see the role of NUP153 in the transport of PP2A and ST into the nucleus as our prospective plan. The role of ST has already been shown to interact with PP2A which ultimately aids in execution of apoptosis in cells. We constructed HA tagged version of NUP153 in mammalian expression vector pCMV-HA in order to go for immunoprecipation assay which would help us confirm the interaction between NUP153 and ST/ PP2A.

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